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<b>13. ABSTRACT (Maximum 200 Words)</b> The five-year survival rate for individuals with a primary breast tumor is 97%. In contrast, the five-year survival rate of individuals with breast cancer metastasis is 23%. Clearly, understanding the molecular mechanisms of metastasis are essential for the prediction and prevention of metastatic disease. Studies of the chromatin protein Heterochromatin Protein 1-alpha (HP1 <sup>Hsa</sup> ) will provide insights on the molecular changes that accompany breast cancer metastasis. HP1 <sup>Hsa</sup> is down-regulated in highly invasive/metastatic breast cancer cells compared with poorly invasive/non-metastatic breast cancer cells. This down-regulation is also observed in metastatic patient tissues. We have determined that transcriptional down-regulation of HP1 <sup>Hsa</sup> in highly invasive/metastatic breast cancer cells occurs through an E-box element in the promoter region. To determine how levels of HP1 <sup>Hsa</sup> regulate invasive properties we have expressed HP1 <sup>Hsa</sup> in highly invasive/metastatic breast cancer cells and knocked down levels of HP1 <sup>Hsa</sup> in poorly invasive/metastatic breast cancer cell lines. Our results demonstrate that addition of HP1 <sup>Hsa</sup> into highly invasive cells reduces their invasive potential. Furthermore, HP1 <sup>Hsa</sup> dimerization is required for this reduction. Subsequent studies will be aimed at changes in gene expression that correlate with altered invasion.				
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## INTRODUCTION

Breast cancer accounts for approximately 30% of all diagnosed cancer and is the most common malignancy in women. The lethality of breast cancer is mainly attributed to its ability to metastasize throughout the body. The five-year survival rate for individuals with metastatic breast cancer is 23%, in contrast to 95% for those with non-metastatic breast cancer (American Cancer Society). Although there has been significant progress made in the identification and understanding of breast cancer over recent years, there remains a need for the identification of molecular markers that definitively distinguish poorly invasive/non-metastatic tumors from highly invasive/metastatic tumors. To identify such prognostic/predictive markers, a clearer understanding of the progression of the metastatic disease state is required.

We have identified a molecular marker that is a candidate breast cancer metastasis suppressor. This marker, termed Heterochromatin Protein 1 ( $HP1^{Hsc}$ ), is an evolutionarily conserved non-histone chromosomal protein (1, 3, 7).  $HP1$  primarily localizes to centric heterochromatin where it plays a role in chromosome segregation and silencing of genes brought into juxtaposition with heterochromatin (4, 6).  $HP1$  also localizes within the gene-rich euchromatic regions of the genome where it is proposed to play a role in gene regulation (1, 17). We discovered that  $HP1^{Hsc}$ , one of three  $HP1$  proteins in humans, was significantly down-regulated in highly invasive/metastatic breast cancer cells compared with poorly invasive/non-metastatic breast cancer cells (9). This observation was specific for  $HP1^{Hsc}$ , and not the other two  $HP1$  family members. We discovered a similar correlation with  $HP1^{Hsc}$  levels in breast cancer patients:  $HP1^{Hsc}$  was abundant in the nuclei of cells from primary breast tumors, but dramatically reduced in cells of metastatic tissue (9). Given the role of  $HP1$  proteins in gene regulation (1, 13, 14), we hypothesize that down-regulation of  $HP1^{Hsc}$  alters the expression of genes involved in invasion and/or metastasis.

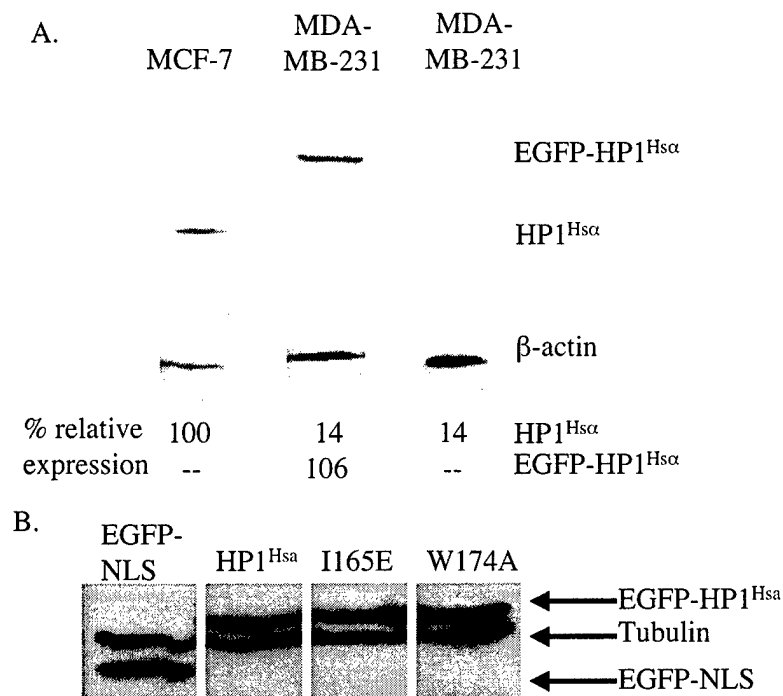
## BODY

Each task and progress on that task is discussed below. All tasks are identical to those stated in the approved STATEMENT OF WORK in the original proposal.

### **Task 1: Determine the consequences of $HP1^{Hsc}$ expression on tumor metastasis markers and global transcriptional expression in human breast cancer cells.**

This task is based on the observation that poorly invasive/non-metastatic breast cancer cells (MCF-7) have levels of  $HP1^{Hsc}$  similar to most cell types, whereas, highly invasive/metastatic cells (MDA-MB-231) have low levels of  $HP1^{Hsc}$  (9). Given the role of  $HP1^{Hsc}$  in gene regulation, we hypothesize that altering levels of  $HP1^{Hsc}$  results in changes in gene expression. To experimentally address this hypothesis we proposed to modulate the levels of  $HP1^{Hsc}$  in both MCF-7 and MDA-MB-231 cells and assay for changes in gene expression. An adenovirus delivery system was utilized to increase levels of  $HP1^{Hsc}$  in MDA-MB-231 cells. This system was used due to our inability to isolate MDA-MB-231 cells that stably expressed a tagged  $HP1^{Hsc}$  transgene. Transient

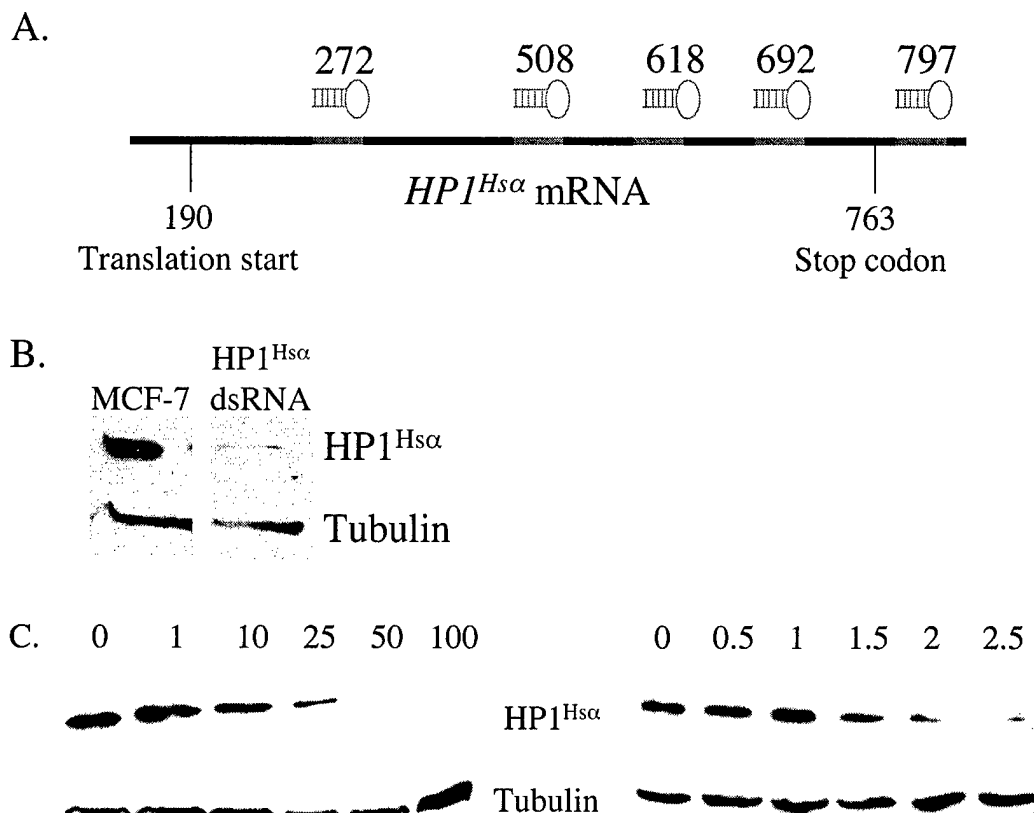
transfection with *HP1<sup>Hsa</sup>*-containing plasmids was not an option due to the low transfection efficiencies (2-5%) obtained with this cell line. In contrast, adenovirus infection results in up to 100% of the cells receiving the transgene (based on EGFP scoring). We constructed adenoviruses containing wild type and mutant *HP1<sup>Hsa</sup>* transgenes tagged at the amino terminus with EGFP. From our work on *Drosophila* HP1 (12) and the work of others on mammalian and *S. pombe* HP1 proteins (11, 19), amino terminal tags do not appear to interfere with HP1 function. Western analysis demonstrated that MDA-MB-231 cells infected with *HP1<sup>Hsa</sup>* adenoviruses express levels of *HP1<sup>Hsa</sup>* protein similar to that of MCF-7 cells at 48 hours post infection (Figure 1A). We have demonstrated that expression of *HP1<sup>Hsa</sup>* in MDA-MB-231 reduced *in vitro* invasion by 30% relative to controls (see Task 3 below). We are currently performing RT-PCR analysis with primers for 20 genes known to be involved in invasion and metastasis. We anticipate that genes such as *E-cadherin*, which promote an invasive phenotype, will decrease in expression, while genes such as *MMP-1*, which inhibit an invasive phenotype, will increase in expression upon expression of the *EGFP-HP1<sup>Hsa</sup>* transgene.



**Figure 1. Protein expression of adenoviral constructs in MDA-MB-231 cells.** (A) Western analysis of EGFP- *HP1<sup>Hsa</sup>* protein levels 24 hour post infection compared to endogenous *HP1<sup>Hsa</sup>* protein levels in uninfected MDA-MB-231 cells and MCF-7 cells (anti-*HP1<sup>Hsa</sup>* antibody from Upstate Biotechnology). Anti-β-actin antibodies (U. of Iowa Hybridoma Core) are used to detect β-actin as a loading control). (B) Western analysis showing similar expression of EGFP-NLS, *HP1<sup>Hsa</sup>*, I165E, and W174A 24 hours post infection.

As a counter experiment to the *EGFP-HP1<sup>Hsa</sup>* expression studies described above, we are also performing RNAi experiments to knock-down the levels of HP1<sup>Hsa</sup> in MCF-7 cells. We generated four RNAi plasmid constructs directed to different regions within the *HP1<sup>Hsa</sup>* mRNA. These constructs were tested for their ability to knock-down HP1<sup>Hsa</sup> in kidney 293T cells. These cells were used because they have a high transfection efficiency compared with MCF-7 cells. Western analysis demonstrated that two of the four constructs (618 and 272, Figure 2A) caused a 90% knock-down of HP1<sup>Hsa</sup> compared to untransfected and GFP transfected control samples 48 hours post transfection (data not shown). The *HP1<sup>Hsa</sup>* sequences within the two successful constructs were subsequently cloned into adenoviral vectors. Infection of MCF-7 cells with the resulting adenoviruses demonstrated a 95% knock-down at 48 post infection relative to uninfected MCF-7 cells (Figure 2C and D). We are currently in the process of comparing levels of knock-down with an adenoviral siGFP construct directed against GFP as a non-specific control. We are also performing *in vitro* invasion assays to determine whether MCF-7 cells gain invasive ability upon knock-down of HP1<sup>Hsa</sup>. To examine changes in gene expression, RNA from MCF-7 cells infected with a control RNAi construct and the *HP1<sup>Hsa</sup>* RNAi construct will be isolated for microarray analysis (HG-U133, Affymetrix). The University of Iowa DNA Core Facility has experience processing Affymetrix microarray chips. Furthermore, our laboratory has performed a similar microarray analysis on wild type and HP1 mutant *Drosophila*. The microarray analysis on HP1<sup>Hsa</sup> knock-down cells will allow us to determine genes that might be involved in the metastatic process in breast cancer cells.

Simultaneous with the generation of the plasmid/viral constructs used for HP1<sup>Hsa</sup> knock-down, we also purchased an siRNA Pool (Dharmacon) containing short dsRNAs to four regions of *HP1<sup>Hsa</sup>* mRNA. These short dsRNA molecules were initially used for transfection in HeLa and 293T cells that have a high efficiency of transfection. Such experiments gave rise to 99% knock-down of HP1<sup>Hsa</sup> compared with cells transfected with a missense dsRNAi as a control (data not shown). Attempts to achieve HP1<sup>Hsa</sup> knock-down in MCF-7 cells by transfection of the HP1<sup>Hsa</sup> dsRNA pool was initially met with difficulties, most likely due to the low transfection efficiencies of MCF-7 cells. However, upon trying the seventh transfection reagent (Lipofectamine 2000), success was observed. We obtain 95% HP1<sup>Hsa</sup> knock-down relative to untransfected and missense controls (Figure 2B and data not shown). Thus, we currently have two independent methods for achieving knock-down of HP1<sup>Hsa</sup> in MCF-7 cells. We are moving forward with the viral constructs rather than the Dharmacon siRNAs since the adenoviral constructs are more cost efficient.



**Figure 2. Knock-down of *HP1<sup>Hsa</sup>* in MCF-7 cells.** (A) Diagram of *HP1<sup>Hsa</sup>* mRNA with regions corresponding to siRNA constructs labeled. (B) Western analysis of *HP1<sup>Hsa</sup>* levels 48 hours after transfection of MCF-7 cells with Dharmacon dsRNAi. *HP1<sup>Hsa</sup>* was detected with monoclonal antibodies specific for *HP1<sup>Hsa</sup>* (Upstate Biotechnology); tubulin was detected with anti-tubulin antibodies (U. of Iowa Hybridoma Core) as a loading control. (C) Western analysis of *HP1<sup>Hsa</sup>* levels 48 hours after infection with adenovirus containing construct 618. Antibodies are as described for (B). Numbers above the lanes correspond to the microliters of virus applied to the cell culture (D) Western analysis of *HP1<sup>Hsa</sup>* levels 48 hours after infection with adenovirus containing construct 272. Antibodies are as described for (B). Numbers above the lanes correspond to microliters of virus applied to the cell culture.

**Task 2: Determine the molecular mechanisms of *HP1<sup>Hsa</sup>* down-regulation in human breast cancer invasion/metastasis.**

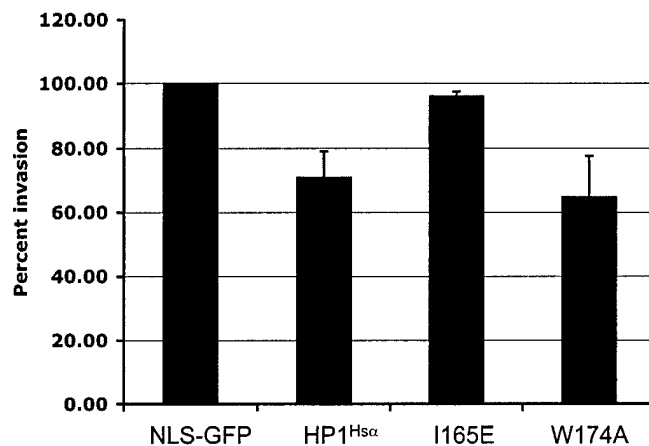
The rationale for this task is that *HP1<sup>Hsa</sup>* mRNA and *HP1<sup>Hsa</sup>* protein levels in MDA-MB-231 cells are reduced 40% and 80%, respectively, compared to that of MCF-7 cells (9). We concluded that one component of the down-regulation acted at the transcriptional

level. Initially, we sequenced fragments of the *HP1<sup>Hsa</sup>* gene from MCF-7 and MDA-MB-231 genomic DNA to examine the sequences for differences between the two cell types. No differences were found within the coding region or 150 bp upstream of the transcription start site. In the absence of genetic sequence changes, we examined the promoter region of *HP1<sup>Hsa</sup>* for epigenetic alterations between the two cell lines. DNA methylation is frequently associated with down-regulation of gene expression in cancer cells (5, 16). Bisulfite sequence analysis revealed that the promoter region of *HP1<sup>Hsa</sup>* is hypomethylated in both cell types (performed in collaboration with M. Kladde, Texas A&M). Last, we reasoned that differential regulation between the two cell lines could be due to altered regulation of transcription factors acting within the promoter region. Constructs with varying amounts of promoter sequences fused to a *luciferase* reporter gene were generated. These constructs were transfected into MCF-7 and MDA-MB-231 cells and luciferase expression was measured. The results demonstrated that differential regulation occurs through an E-box consensus element within the promoter region. Details of these experiments have been published (15) (see Appendix).

### **Task 3: Determine the domains of *HP1<sup>Hsa</sup>* required for invasion and metastasis.**

*HP1<sup>Hsa</sup>* has a two-domain structure consisting of an amino chromo domain (CD) and a carboxy chromo shadow domain (CSD). The CD associates with methylated lysine 9 of histone H3 (8) and is thought to be the primary mechanism of *HP1* localization within centric heterochromatin. The CSD homodimerizes (2); this dimerization generates a surface that interacts with a variety of proteins possessing a pentapeptide motif (18). In order to determine the domains of *HP1<sup>Hsa</sup>* involved in metastasis, we generated four mutant constructs: 1) a truncation that leaves just the CD, 2) a truncation that leaves just the CSD, 3) a point mutation, I165E, that disrupts the dimerization of *HP1* (10) and 4) a point mutation, W174A (10), that disrupts interaction with the pentapeptide (18). Due to the high costs associated with viral packaging, only the wild type and two point mutations have been packaged into adenoviruses. Infection with the control nuclear-tagged EGFP construct did not significantly alter invasion when compared with the uninfected MDA-MB-231 cells (data not shown). Viral titers were adjusted to achieve similar amounts of protein production for all constructs by western analysis (Figure 1B). Infection with the wild type *HP1<sup>Hsa</sup>* construct correlated with a 30% reduction in invasion compared with the nuclear-tagged EGFP (Figure 3). A 30% reduction was also observed for the W174A mutation, suggesting that interactions with pentapeptide motif containing proteins do not play a role in regulating invasion. In contrast, the point mutation that disrupts *HP1<sup>Hsa</sup>* homodimerization, I165E, exhibit invasion levels similar to the nuclear-tagged EGFP control. Thus, *HP1<sup>Hsa</sup>* dimerization appears to be essential for the regulation of *in vitro* invasion.





**Figure 3. HP1<sup>Hsc</sup> alters *in vitro* invasion of MDA-MB-231 cells.** MDA-MB-231 cells were infected with adenovirus constructs expressing wild type and mutant HP1<sup>Hsc</sup> and nuclear-tagged EGFP as a control. The I165E mutation disrupts HP1 homodimerization. The W174A mutation disrupts interaction with pentapeptide containing proteins, while allowing homodimerization.

## KEY RESEARCH ACCOMPLISHMENTS

- Generated adenoviral vectors that express nuclear-tagged EGFP and mutant HP1<sup>Hsc</sup> amino terminally tagged with EGFP.
- Demonstrated that adenoviral vectors express stable protein products at 168 hours post infection.
- Demonstrated that two of the four RNAi plasmid constructs effectively knocked-down expression of HP1<sup>Hsc</sup> in HeLa and 293T cells.
- Demonstrated that two adenoviral RNAi constructs effectively knock-down expression of HP1<sup>Hsc</sup> in MCF-7 breast cancer cells.
- Demonstrated that short dsRNA oligos effectively knock-down levels of HP1<sup>Hsc</sup> in MCF-7 breast cancer cells.
- Identified an E-box consensus element as a major contributor to the differential regulation of HP1<sup>Hsc</sup> observed between MCF-7 and MDA-MB-231 cells.
- Addition of exogenous HP1<sup>Hsc</sup> to MDA-MB-231 cells (to levels comparable to MCF-7 HP1<sup>Hsc</sup> expression) correlates with a reduction in *in vitro* invasion.
- A single point mutation that disrupts dimerization, eliminates the ability of HP1<sup>Hsc</sup> to cause a reduction in *in vitro* invasion.

## REPORTABLE OUTCOMES

### Publication

Norwood, L.E., Grade, S.K., Cryderman, D.E., Hines, K.A., Furiasse, N., Toro, R. Li, Y., Dhasarathy, A., Kladde, M.P., Hendrix, M.J.C. and Kirschmann, D.A. and L.L. Wallrath (2004). Conserved properties of HP1<sup>Hsc</sup>. Gene (In Press).

### Poster Abstracts

Norwood, L.E., Wright, L., Margaryan, N.M, Cryderman, D.E., Hendrix, M.J.C., Kirschmann, D.A. and L.L. Wallrath (2004). Heterochromatin Protein 1: Development of a novel breast cancer metastasis marker. University of Iowa Review of the 2003 26<sup>th</sup> San Antonio Breast Cancer Symposium.

Moss, T.J., Norwood, L., Ferraro, D., Sloat, S., Hitchler, M.J. Hendrix, M.J.C., Kirschmann, D.A. and L.L. Wallrath. Structural and functional analysis of Heterochromatin Protein 1 in breast cancer cells. University of Iowa Review of the 2003 26<sup>th</sup> San Antonio Breast Cancer Symposium.

## CONCLUSIONS

This year we have made significant progress on all three tasks of the original proposal. We have determined that the differential regulation of HP1<sup>Hsc</sup> between MCF-7 cells and MDA-MB-231 cells occurs through an E-box consensus element in the promoter region. Several transcription factor families have members that bind to this consensus sequence. Subsequent research to identify the factor involved at the HP1<sup>Hsc</sup> promoter is likely to be complex and beyond the scope of this proposal. Therefore, we have finished Task 2, which has resulted in one publication.

We are now successfully able to modulate levels of HP1<sup>Hsc</sup> through adenoviral delivery of wild type and mutant HP1<sup>Hsc</sup> transgenes and siRNA constructs. This success sets the stage for exciting experimentation to examine *in vitro* invasion, gene expression and a domain structure/functional analysis of HP1<sup>Hsc</sup>. We have already demonstrated that wild type HP1<sup>Hsc</sup> when introduced into MDA-MB-231 cells reduces *in vitro* invasion by 30% compared to nuclear EGFP controls. This reduction depends on the ability of HP1<sup>Hsc</sup> to dimerize. We anticipate that a minimum of two additional publications will result within the next year. One publication will focus on the effects of expressing HP1<sup>Hsc</sup> in MDA-MB-231 cells and the other will focus on the knock-down of HP1<sup>Hsc</sup> in MCF-7 cells, both publications targeted to journals such as Cancer Research.

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## APPENDICES

1. One Publication
2. Two Poster Abstracts

Conserved properties of HP1<sup>Hsa</sup>

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**Abstract**

Heterochromatin protein 1 Hsa (HP1<sup>Hsa</sup>) is one of three human proteins that share sequence similarity with *Drosophila* HP1. HP1 proteins are enriched at centric heterochromatin and play a role in chromatin packaging and gene regulation. In humans, HP1<sup>Hsa</sup> is down-regulated in highly invasive/metastatic breast cancer cells, compared to poorly invasive/non-metastatic breast cancer cells. To gain insight into this differential regulation, we have cloned the HP1<sup>Hsa</sup> gene and characterized its genomic region. HP1<sup>Hsa</sup> is located on human chromosome 12q13.13, 589 bp upstream of the divergently transcribed *hnRNPA1* gene. Analysis of the promoter region revealed that differential regulation of HP1<sup>Hsa</sup> between the two types of breast cancer cells is lost upon mutation of an USF/c-myc transcription factor binding site located 172 bp upstream of the predicted HP1<sup>Hsa</sup> transcription start site. These findings provide insights into the down-regulation of HP1<sup>Hsa</sup> in highly invasive/metastatic breast cancer cells. To examine the functional properties of HP1<sup>Hsa</sup>, experiments were performed using *Drosophila melanogaster* as a genetic system. When human HP1<sup>Hsa</sup> was expressed in transgenic *Drosophila*, silencing of reporter genes inserted at centric and telomeric locations was enhanced. Furthermore, expression of HP1<sup>Hsa</sup> rescued the lethality of homozygous *Su(var)2-5* mutants lacking HP1. Taken together, these results demonstrate the participation of HP1<sup>Hsa</sup> in silent chromatin formation and that HP1<sup>Hsa</sup> is a functional homologue of *Drosophila* HP1.

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**Keywords:** Breast cancer metastasis; *Drosophila*; Gene silencing; Heterochromatin

**1. Introduction**

Heterochromatin protein 1 (HP1) was first discovered in *Drosophila melanogaster* and has since been found in a variety of eukaryotes from *Schizosaccharomyces pombe* to humans (Eissenberg and Elgin, 2000). *Drosophila*, mice and humans have three HP1 family members. All HP1 proteins

have a conserved amino domain termed the chromo domain (CD) and a carboxy domain termed the chromo shadow domain (CSD), separated by a less conserved hinge region (Eissenberg and Elgin, 2000). The HP1 CD binds to methylated lysine nine of histone H3; this interaction is important for HP1 localization at centric regions of chromosomes (Brehm et al., 2004).

The HP1 CSD homodimerizes, forming a site of interaction for several nuclear proteins possessing a pentapeptide motif (PxVxL), including the HP1 CSD itself (Cowieson et al., 2000; Smothers and Henikoff, 2000). The hinge region of some HP1 family members interacts with RNA and histone H1 (Nielsen et al., 2001; Muchardt et al., 2002). Thus, HP1 can be thought of as a bridging molecule that links various nuclear proteins to the chromosome.

The known functions of HP1 proteins are largely based on genetic data. HP1 was identified in *Drosophila* muta-

**Abbreviations:** bp, base pair(s); kb, kilobase(s); hp1, heterochromatin protein 1; CD, chromo domain; CSD, chromo shadow domain; CAF1, chromatin assembly factor 1; µg, microgram; EGFP, enhanced green fluorescent protein; PCR, polymerase chain reaction; 5'RACE, 5' rapid amplification of cDNA ends; CMV, cytomegalovirus; USF, upstream stimulatory factor.

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genesis screens for modifiers of heterochromatic gene silencing (Weiler and Wakimoto, 1995). Mutations in the *Drosophila* gene encoding HP1, *Su(var)2-5*, are homozygous lethal; heterozygotes show suppression of silencing of genes placed near heterochromatin, implying a role for HP1 in chromatin packaging. In *S. pombe*, mutations in the HP1-like protein Swi6 lead to chromosome segregation defects (Ekwall et al., 1995). Information gleaned from studies of HP1 in model organisms allows one to infer the function of HP1 homologs in mammalian cells, where genetic assays are not currently available.

In humans, *HP1<sup>Hsa</sup>* is specifically down-regulated in highly invasive/metastatic breast cancer cells compared with poorly invasive/non-metastatic breast cancer cells, both at mRNA and protein levels (Kirschmann et al., 2000). Consistent with these cell culture phenotypes, staining of breast cancer tissue samples with antibodies to *HP1<sup>Hsa</sup>* showed that *HP1<sup>Hsa</sup>* was decreased in distant metastases compared to primary tumor tissues (Kirschmann et al., 2000). In this study, we identify sequences within the *HP1<sup>Hsa</sup>* promoter region that are responsible for differential expression in metastatic vs. nonmetastatic breast cancer cell lines. In addition, we show here that *HP1<sup>Hsa</sup>* is a functional homolog of *Drosophila* HP1.

## 2. Materials and methods

### 2.1. Isolation of genomic clones

A lambda genomic library made from the whole placenta of a 27-year-old healthy Caucasian female (Clontech) was screened to recover *HP1<sup>Hsa</sup>* genomic clones. 1.5 × 10<sup>6</sup> independent clones were screened using standard techniques. *HP1<sup>Hsa</sup>* cDNA was random prime labeled with <sup>32</sup>P-dATP (Amersham Multiprime labeling kit) and used for hybridization. Southern analysis was used to identify fragments within the clones containing *HP1<sup>Hsa</sup>*. These fragments were isolated and sequenced to determine intron/exon boundaries of the *HP1<sup>Hsa</sup>* gene.

### 2.2. 5'RACE

RNA was isolated from MCF-7 cells using TRIzol reagent (Life Technologies). RNA was amplified according to the 5'RACE System (Life Technologies) using a primer specific to a region 34–59 bp downstream of the stop codon. cDNA was amplified at 50 °C instead of the typical 42 °C to minimize secondary structure. The cDNA was PCR amplified using the abridged anchor primer (Life Technologies) specific to the C-tailed cDNA and a primer specific to the fifth exon. An additional extension cycle at 72 °C for 3 min was added at the end of the PCR cycles. The PCR products were cloned (TA cloning system, Invitrogen) and sequenced.

### 2.3. Cells and culture conditions

MCF-7 cells were kindly supplied by Dr. F. Miller (Karmanos Cancer Institute, Detroit, MI). MDA-MB-231 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cell lines were maintained as previously described (Kirschmann et al., 1999).

### 2.4. Bisulfite genomic sequencing

Genomic DNA was isolated and analyzed by bisulfite genomic sequencing as previously described (Kladde et al., 1996). PCR products, amplified from bisulfite-deaminated DNA using JumpStart *Taq* DNA polymerase (Sigma), were purified and subjected to primer extension as described (Kladde et al., 1996), except that the final concentrations of dNTPs (A, C, T) and ddGTP were 50 and 150 μM, respectively. Exclusion of dGTP from the PCR product primer extension reactions generated high termination efficiencies (>96%) (Kladde et al., 1996) at template cytidines (nucleotides that were <sup>5</sup>meC residues in vivo).

### 2.5. Plasmid constructs

Fragments of the *HP1<sup>Hsa</sup>* promoter region (positions –600, –466, –418, –286, –166, and –110 bp relative to transcription start at +1) fused to exon one (+143) were cloned into the pGL3-Basic vector (Promega). Forward primers used for generating the deletion constructs were as follows: –600 bp primer 5'-GCAGAAAGGAGC-GAGCTCACGAACGTATC-3', –466 bp primer 5'-CCTGCTATTGAGCTCTGGTGCCACATTGC-3', –418 bp primer 5'-GGTCGTTCTACGAGCTCTCCACC-3', –286 bp primer 5'-CTTCCACGAGCTCATATTACAGT-CAAG-3', –166 bp primer 5'-GTAAATGCGCA-GCTCTGCGCA-3', –110 bp primer 5'-CGTGAAATG-GAGCTCAGGAGTAGG-3'. The reverse primer used for all of the *HP1<sup>Hsa</sup>* promoter deletion constructs recognizes *HP1<sup>Hsa</sup>* exon one and the pGL3-Basic vector: 5'-AGATCTCGAGCCCGGGATTGAGAGTGATCA-3'. The mutant transcription factor binding sites were generated using the QuikChange site-directed mutagenesis kit (Stratagene) and the following primers: δEF1 (mδEF1): 5'-CGCATTAAGAAGTCCCCCCCCCTCTGAGAACACG-3' and 5'-CGTGTCTCAGAGGGGGGGGGAAGTCTTCTTAATGCG-3', c-myc (mmyb): 5'-CGTTTGGCGGGCCCCCCCCCTTGCGCAGAAGG-3' and 5'-CCTTCTGCGCAAGGGGGGGGGGCCGCCAAAACG-3', USF/c-myc site at position –109 (mUSFp): 5'-CCTCTGAGACCCCCCAATGGCGGGCAGGAGTAG-3' and 5'-CTACTCCTGCCCGCCATTGGGGGGGGTCTCAGAGG-3', USF/c-myc site at position –172 (mUSFd): 5'-CTCTTGTGTGACCGGGGGGGAGTAAATGGCG-3' and 5'-CGCCATT-TACTCCCCCCCCGGTCAACAAGAG-3'. The double mutants of mmyb and mUSF1 (mmybUSFp) and mUSF1

159 and mUSF2 (mUSFpUSFd) were also made using the  
160 above primers.

161

## 162 2.6. Transient transfection assays

163 MCF-7 and MDA-MB-231 cells were grown to 80%  
164 confluency. A total of 1 µg of DNA, including 0.5 µg of  
165 promoter construct and 0.5 µg of *CMV-lacZ* (kind gift of  
166 Dr. Andrew Russo), was transfected into the cell lines  
167 using Effectene Transfection Reagent (Qiagen). The cells  
168 were grown for 48 h, collected with Cell Culture Lysis  
169 Reagent (Promega), and assayed for luciferase and β-  
170 galactosidase expression. Luciferase expression was mea-  
171 sured using Luciferase Assay Substrate (Promega) to  
172 monitor expression from *HP1<sup>Hsx</sup>* promoter constructs. β-  
173 Galactosidase expression was measured using Galacto-  
174 Light Plus System (Applied Biosystems) to normalize  
175 for transfection efficiency. Light units were measured on  
176 a 96-well plate luminometer (Dynex). Normalized lucifer-  
177 ase light unit measurements were set relative to light unit  
178 measurements obtained for a promoter construct contain-  
179 ing 4 kb of *HP1<sup>Hsx</sup>* upstream sequences, including exon  
180 one. This 4-kb construct gives uniform low levels of  
181 expression in both cell lines. These data were analyzed  
182 using the Microsoft Excel two samples unequal variance  
183 Student's *t*-test.

184

## 185 2.7. P-element construct and *Drosophila* germ line 186 transformation

187 *HP1<sup>Hsx</sup>* was fused in frame with *EGFP* and inserted  
188 into the P-element vector pCaSpeRhs-act ([http://thummel.  
189 genetics.utah.edu/vector%20map%20htmls/pcasper-hs-act.  
190 html](http://thummel.genetics.utah.edu/vector%20map%20htmls/pcasper-hs-act.html)) containing an *hsp70* promoter to drive expression of  
191 the fusion gene and a mini-*white<sup>+</sup>* gene for selection of  
192 transformants. To generate an untagged *HP1<sup>Hsx</sup>* construct,  
193 *HP1<sup>Hsx</sup>* cDNA was inserted into the P-element vector  
194 pCaSpeR-hs-act. Both resulting P-element constructs were  
195 independently injected into *y, w<sup>67c23</sup>* *Drosophila* embryos,  
196 along with P-turbo helper plasmid encoding transposase,  
197 according to standard germ-line transformation procedures.  
198 Daily heat-shock treatments lead to an estimated threefold  
199 higher expression of *HP1<sup>Hsx</sup>*-EGFP than the endogenous  
200 HP1 protein as judged by Western analysis (data not  
201 shown).

202

## 203 2.8. *Drosophila* genetics

204 All *Drosophila* stocks were raised on standard corn meal  
205 sucrose media (Shaffer et al., 1994) at 25 °C. Females with  
206 the genotype *P[w<sup>+</sup>, hsp70-HP1<sup>Hsx</sup>]; Su(var)2-5<sup>04</sup>/CyO*,  
207 *GFP* were crossed to males of the genotype *Su(var)2-5<sup>02</sup>/*  
208 *CyO, GFP*. Crosses were heat shocked at 37 °C for 45 min  
209 daily. Rescue of lethality was indicated by the presence of  
210 straight winged adults, representing the genotype *P[w<sup>+</sup>,*  
211 *hsp70-HP1<sup>Hsx</sup>]; Su(var)2-5<sup>04</sup>/Su(var)2-5<sup>02</sup>*.

## 2.9. Immunostaining of polytene chromosomes

Third instar larvae were heat shocked at 37 °C for 1 h and  
allowed to recover at room temperature for 2 h. Salivary  
glands were dissected, fixed, squashed and stained with a  
monoclonal antibody to HP1 (C1A9) and a polyclonal  
antibody to GFP (Molecular Probes) according to published  
procedures (Platero et al., 1995).

## 2.10. Northern analysis

RNA for Northern analysis was isolated from third instar  
larvae after heat shock at 37 °C for 1 h according to  
published procedures (Wallrath et al., 1990). Levels of  
mRNA produced by the heterochromatic transgenes were  
measured by hybridization with *barley* cDNA sequences  
fused to the *hsp26* transgene and labeled with <sup>32</sup>P-dATP  
(Amersham) using random prime labeling (Amersham). The  
*rp49* cDNA was used as a control for RNA loading.

## 3. Results

### 3.1. Structure of the *HP1<sup>Hsx</sup>* genomic region

*HP1<sup>Hsx</sup>* is down-regulated in highly invasive/metastatic  
breast cancer cells in comparison to poorly invasive/non-  
metastatic breast cancer cells (Kirschmann et al., 2000). To  
better understand the mechanism of *HP1<sup>Hsx</sup>* down-regula-  
tion, we have determined the structure of the *HP1<sup>Hsx</sup>*  
genomic region, including the promoter region (Fig. 1).  
Clone F2-10 contains exons two and three surrounded by  
repetitive sequences typically found in introns. This clone  
spans a region approximately 9 kb upstream of exon two to  
300 bp downstream of exon three. Exon two contains the  
methionine translation start codon. Clone F2-11 contains  
exons three, four, and five. A second screen, using sequen-  
ces corresponding to exon one and 150 bp upstream as a  
probe, identified four clones containing the *HP1<sup>Hsx</sup>* prom-  
oter region. Clone 3-4 contains 11 kb of the *HP1<sup>Hsx</sup>* promoter  
region in addition to exon one that is 5' untranslated  
sequence. Taken together, *HP1<sup>Hsx</sup>* is encoded by five exons  
spanning 38 kb.

5'RACE was performed using a primer to *HP1<sup>Hsx</sup>*  
(positions +799 to +824) to identify the potential  
transcription start site. Three products, having their 5'  
ends within 22 bp of each other, were identified. We  
designated +1 as the 5' end of the longest 5'RACE  
product, extending exon one of *HP1<sup>Hsx</sup>* an additional 54  
bp upstream as compared to the NCBI CBX5 cDNA  
sequence NM\_012117 (Fig. 1).

A bioinformatics analysis was performed on *HP1<sup>Hsx</sup>*  
promoter region sequences. Using MatInspector V2.2  
<http://transfac.gbf.de/TRANSFAC/>) at stringent conditions  
(core sim 1.0, matrix sim 0.95), sequences from -601 to  
+143 were analyzed for known transcription factor binding

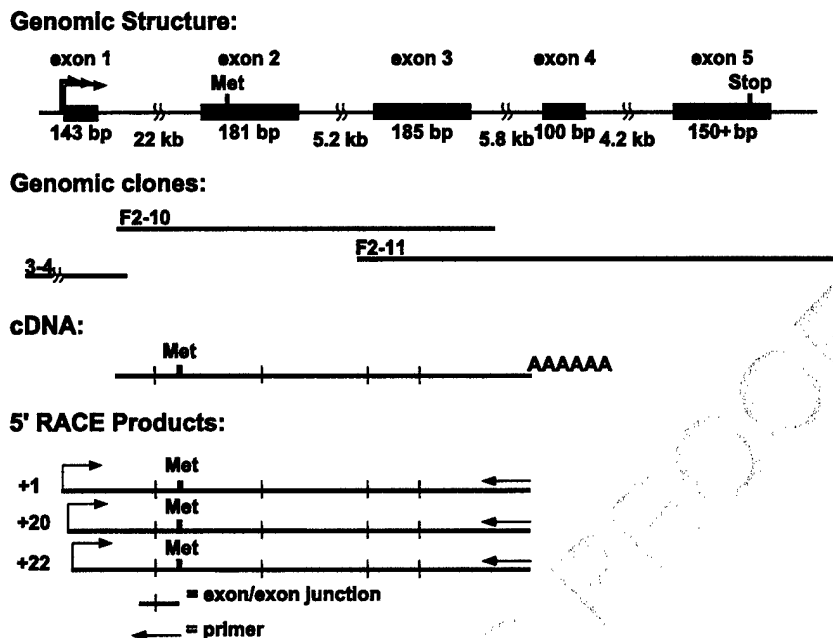


Fig. 1. The genomic structure of the human *HP1<sup>Hsa</sup>* gene with the lengths of each exon and intron in bp or kb are shown. Transcription start sites are assigned on the basis of 5'RACE products. The translation methionine start (ATG) and stop are shown. The genomic clones are represented by lines under the corresponding genomic regions. Three clones obtained using 5'RACE start within 54 bp upstream of the cDNA sequences previously published in Genbank. The clone containing the most 5' sequence was designated +1; additional clones start at +20 and +22.

264 sites. Using these criteria, 37 binding sites, some over-  
 265 lapping with each other, were identified within this region,  
 266 but no TATA box was identified. The lack of a TATA box is  
 267 consistent with having multiple transcription start sites  
 268 (Gum et al., 2003).  
 269 The *hnRNPA1* gene is divergently transcribed, starting at  
 270 position -589 bp upstream of the *HP1<sup>Hsa</sup>* transcription start  
 271 (Fig. 3A). Although the promoter regions of *hnRNPA1*  
 272 and *HP1<sup>Hsa</sup>* are in close proximity, they appear to be  
 273 independently regulated in breast cancer cells. Unlike the  
 274 different levels of *HP1<sup>Hsa</sup>* observed in the two breast  
 275 cancer cell lines, *hnRNPA1* levels are unchanged be-  
 276 tween MDA-MB-231 and MCF-7 cells (data not shown).  
 277 DNase I footprinting of the region between the *hnRNPA1*  
 278 and *HP1<sup>Hsa</sup>* genes was performed using HeLa cells  
 279 (Biamonti et al., 1993). Six potential transcription factor  
 280 binding sites found in our bioinformatics analysis of the  
 281 *HP1<sup>Hsa</sup>* promoter region correspond to the DNase I  
 282 footprints previously identified, including two SP1 sites,  
 283 two CAAT boxes, a CREB/c-jun site, two USF/c-myc  
 284 sites, and a c-myc site (Biamonti et al., 1993) (Fig. 3A).  
 285 A  $\delta$ EF1 site within the promoter region is also of  
 286 interest. The human AREB6 repressor protein that binds  
 287 to the  $\delta$ EF1 site is up-regulated in highly invasive/  
 288 metastatic cell lines compared to poorly invasive/non-  
 289 metastatic cell lines (Kirschmann et al., 1999). Therefore,  
 290 the  $\delta$ EF1 site was analyzed as a candidate regulatory  
 291 element (Fig. 3A).

### 3.2. Mechanism of *HP1<sup>Hsa</sup>* differential regulation

294 The well-characterized breast cancer cell lines MDA-  
 295 MB-231, which is highly invasive/metastatic, and MCF-7,  
 296 which is poorly invasive/non-metastatic, were used to de-  
 297 termine the mechanism of differential regulation of *HP1<sup>Hsa</sup>*.  
 298 One possible explanation for differential expression of  
 299 *HP1<sup>Hsa</sup>* is that a mutation in the *HP1<sup>Hsa</sup>* gene within  
 300 MDAMB-231 cells results in reduced expression. The  
 301 coding region, splice junctions, portions of the introns,  
 302 and 150 bp of the promoter region of *HP1<sup>Hsa</sup>* from MDA-  
 303 MB-231 and MCF-7 cells were sequenced and compared.  
 304 No differences between the *HP1<sup>Hsa</sup>* genomic sequences of  
 305 the two cell lines were found. Therefore, differential regu-  
 306 lation of *HP1<sup>Hsa</sup>* is not likely due to mutations within the  
 307 sequenced regions of *HP1<sup>Hsa</sup>*.

308 As a second possibility to explain the differential expres-  
 309 sion, we investigated the DNA methylation status of *HP1<sup>Hsa</sup>*  
 310 in both cell types. In many cases transcriptional regulation  
 311 in cancer cells is under control of DNA methylation,  
 312 particularly for genes near CpG islands (Dallol et al.,  
 313 2003). A CpG island within exon one of the *hnRNPA1* gene  
 314 (-482 to -899 from *HP1<sup>Hsa</sup>* transcription start) was  
 315 identified using CpGReport <http://www.ebi.ac.uk/>. Both  
 316 strands of the *HP1<sup>Hsa</sup>* promoter region (bases -900 to  
 317 +168), including the CpG island, were subjected to bisulfite  
 318 sequencing to determine DNA methylation of *HP1<sup>Hsa</sup>* in the  
 319 MDA-MB-231 and MCF-7 cell lines (Fig. 2 shows the



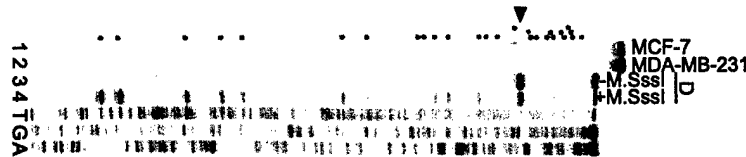


Fig. 2. Absence of detectable 5-methylcytosine ( $^{5\text{mC}}$ ) within the  $HP1^{Hsa}$  promoter region in MCF-7 and MDA-MB-231 cells. DNA from both cell lines (lanes 1 and 2) was subjected to a sensitive variation of genomic bisulfite sequencing that is able to detect low levels of  $^{5\text{mC}}$ . Analysis of sequences from  $-220$  to  $+168$  of the  $HP1^{Hsa}$  promoter is shown. Plasmid DNA containing the  $HP1^{Hsa}$  promoter methylated in vitro by  $M. SssI$  (lane 4) provides a marker for modified CpG sites (filled circles). Since the plasmid was isolated from a  $dcm^+$  strain of *E. coli*, methylation at a  $dcm$  site (arrow head) was also detected (lanes 3 and 4). Methylation at the  $dcm$ - and  $M. SssI$ -modified sites demonstrates the signal intensity that is commensurate with high and moderate levels of DNA methylation, respectively. Thus, MCF-7 and MDA-MB-231 cells do not have detectable levels of  $^{5\text{mC}}$ . Reactions carried out on purified plasmid DNA are labeled D.

methylation status of bases  $-220$  to  $+168$ ; bases  $-900$  to  $-220$  are not shown). Limited DNA methylation, if any, was observed in either cell type, and the methylation status was unchanged between the two cell lines throughout the  $HP1^{Hsa}$  promoter region and exon one, including the CpG island within *hnRNPA1*. Thus, methylation does not appear to be involved in the differential regulation of  $HP1^{Hsa}$ . As a third explanation for the differential regulation, we hypothesized that differential expression might arise through different interactions between transcription factors and *cis*-acting DNA elements of the  $HP1^{Hsa}$  promoter in the two cell types. Fragments of the  $HP1^{Hsa}$  promoter, including untranslated exon one, were cloned upstream of a luciferase reporter gene. These constructs were co-transfected into MDAMB-231 and MCF-7 cells along with a plasmid containing the *CMV* promoter driving a *lacZ* reporter gene. Expression of luciferase and  $\beta$ -galactosidase was measured in light units. Luciferase expression was normalized to  $\beta$ -galactosidase expression to account for differences in transfection efficiency. Levels of luciferase above background were observed from all constructs, indicating that the cloned promoter region possessed transcriptional activity. A 5.6-fold difference in luciferase activity was observed between the MDA-MB-231 and MCF-7 cell lines for the largest construct,  $-600/+143$ , containing 13 sequences between the  $HP1^{Hsa}$  and the *hnRNPA1* predicted transcription start sites ( $p$ -value =  $0.0021$ ) (Fig. 3B and C). This difference in expression is similar to that observed for endogenous  $HP1^{Hsa}$  gene between the two cell types (Kirschmann et al., 2000). Deletions that removed successive amounts of 5' sequences showed a reduction in the fold change in expression between the two cell types, suggesting the loss of regulatory sequences (Fig. 3B). In particular, the differences in expression between the  $-166/+143$  construct in the two cell types were barely statistically significant ( $p$ -value =  $0.0608$ ). One caveat of these deletion constructs is

that vector DNA sequences are brought into close proximity of the  $HP1^{Hsa}$  promoter, possibly contributing to regulation.

To better identify elements involved in differential regulation of  $HP1^{Hsa}$  between the two cell types, constructs containing mutations within candidate transcription factor binding sites were analyzed. These constructs allowed for the retention of 600 bp of upstream sequences. Of particular interest was a  $\delta$ EF1 binding site at position  $-125$  (Fig. 3A). This site can be bound by the human homolog of the chicken  $\delta$ EF1 protein, AREB6, a transcriptional repressor (Ikeda et al., 1998) that is up-regulated in highly invasive/metastatic cell lines, including MDA-MB-231, compared to poorly invasive/non-metastatic cell lines, such as MCF-7 (Kirschmann et al., 1999). Thus, the  $\delta$ EF1 binding site was a promising candidate for regulating differential expression of  $HP1^{Hsa}$ . A mutation in the conserved  $\delta$ EF1 site ( $m\delta$ EF1) was constructed by replacing the 4 bp core binding site and 5 bp of surrounding sequence with nine cytosines in the context of the  $-600/+143$  construct. Differential expression between the two cell lines was still observed (2.87-fold difference,  $p$ -value =  $0.0004$ ) (Fig. 3C). Therefore, the  $\delta$ EF1 site does not appear to be involved in differential regulation of  $HP1^{Hsa}$ .

Several additional candidate transcription factor-binding sites were also investigated for their effects on differential regulation. These include a c-myc site within exon one of  $HP1^{Hsa}$  (Fig. 3A). Mutation of this site ( $mmyb$ ), replacing the core binding region and surrounding bases with nine cytosines, retained differential expression (ninefold difference,  $p$ -value =  $0.0124$ ) (Fig. 3C). Furthermore, mutation of two USF/cmyc sites located at positions  $-109$  and  $-172$ , designated USFd (distal) and USFp (proximal), respectively, was tested for effects on expression (Fig. 3A). Mutations in the individual sites ( $mUSFp$  and  $mUSFd$ ), double mutations of both USF/c-myc sites ( $mUSFpUSFd$ ), and mutation of USFp in combination with mutation of the c-myc site

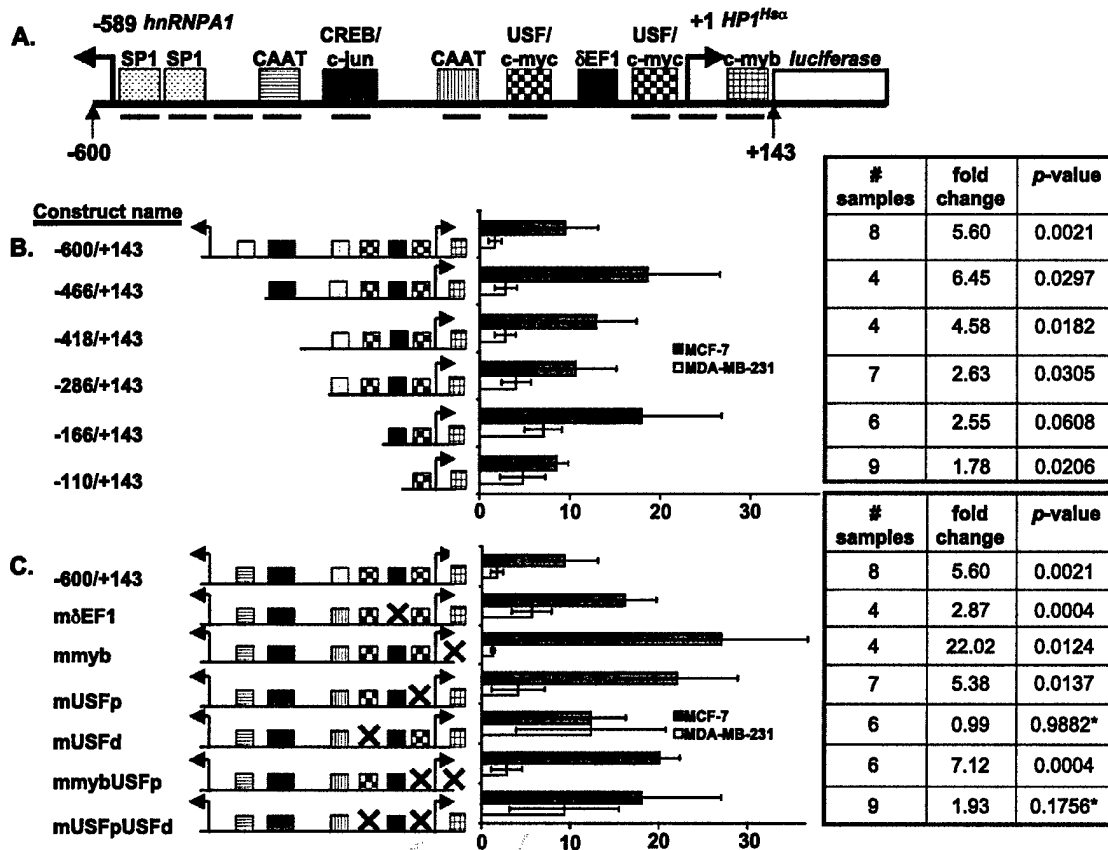


Fig. 3. (A) Diagram of the promoter region between the divergently transcribed *hnRNPA1* and *HP1<sup>Hsa</sup>* genes. Consensus transcription factor binding sites that were previously shown to be footprinted by protein in HeLa cells (Biamonti et al., 1993) are indicated by black lines. Transcription factor binding sites identified by our bioinformatics searches are indicated by the name above the boxes. (B) Results of luciferase assays from 5' deletion constructs containing *HP1<sup>Hsa</sup>* promoter fragments fused to a *luciferase* reporter gene. The construct names indicate the 5' and 3' sequence boundaries. The constructs were transfected into a metastatic/highly invasive cell line, MDA-MB-231 and a nonmetastatic/poorly invasive cell line, MCF-7. The number of samples, fold change between expression in MCF-7 and MDA-MB-231 cells, and *p*-values are indicated for each construct. (C) Results of mutational analysis of the *HP1<sup>Hsa</sup>* promoter constructs. Construct names reflect the site(s) mutated. Asterisks mark *p*-values that show no statistical difference in expression between the two cell lines, indicating a loss of differential regulation.

(mmybUSFp) were examined. All of the mutations replaced the core 4 bp of the transcription factor binding sites and surrounding sequence with eight cytosines. The mUSFp mutation retained differential expression (5.38-fold difference, *p*-value=0.0137) (Fig. 3C). The mmybUSFp double mutant also retained differential expression (7.12-fold difference, *p*-value=0.0004). In contrast, mUSFd eliminated differential expression (0.99-fold difference, *p*-value=0.9882). Consistent with this finding, mUSFpUSFd disrupted differential expression (*p*-value=0.1756). Thus, the USFd site at position -172 is required for differential expression of *HP1<sup>Hsa</sup>* between MCF-7 and MDA-MB-231 cell lines.

### 3.3. Human *HP1<sup>Hsa</sup>* functions similar to *Drosophila HP1*

In addition to understanding *HP1<sup>Hsa</sup>* regulation, we also aimed to understand the functional properties of this protein.

*HP1* proteins are highly conserved between species, suggesting related functions. *HP1* was first identified in *Drosophila* where functional studies have been performed, demonstrating a role in gene silencing (Weiler and Wakimoto, 1995). Three human *HP1* family members, *HP1<sup>Hsa</sup>*, *HP1<sup>Hsβ</sup>* and *HP1<sup>Hsγ</sup>*, show a high degree of amino acid sequence identity with *Drosophila HP1*; however, it is unclear which family member is the functional homolog of *Drosophila HP1*. *HP1<sup>Hsγ</sup>* shows 44% overall amino acid sequence identity with *Drosophila HP1*, 60% in the CD, and 38% in the CSD. These values are similar to those of *HP1<sup>Hsa</sup>* showing 44% overall, 65% in the CD, and 43% in the CSD. *HP1<sup>Hsβ</sup>* has slightly greater overall amino acid identity to *Drosophila HP1* than *HP1<sup>Hsa</sup>* (46% vs. 44%). The *HP1<sup>Hsβ</sup>* CD shows slightly more identity with the *Drosophila HP1* CD than the *HP1<sup>Hsγ</sup>* and *HP1<sup>Hsa</sup>* CDs (68% vs. 60% and 65%, respectively). In contrast, the *HP1<sup>Hsβ</sup>* CSD shows less identity to the *Drosophila HP1*

429 CSD than the  $HP1^{Hsa}$  CSD (39% vs. 43%). In sum,  
 430 comparisons of the amino acid sequences of human and  
 431 Drosophila HP1 identified only minor differences in the  
 432 percent identity without immediately suggesting a function-  
 433 al homologue.

434 Another protein feature that might suggest similar function  
 435 between Drosophila HP1 and a human HP1 is the  
 436 chromosome localization pattern. Drosophila HP1 shows  
 437 enrichment at heterochromatic regions and localizes to  
 438 approximately 200 euchromatic sites on larval polytene  
 439 chromosomes (Fanti et al., 2003).  $HP1^{Hsa}$  and  $HP1^{Hs\beta}$   
 440 predominantly localize to centric heterochromatin, showing  
 441 partial overlap with anticentromere antibodies (Minc et al.,  
 442 1999). In contrast,  $HP1^{Hsy}$  localizes to centric heterochro-  
 443 matin and euchromatic regions (Minc et al., 2000). Based on  
 444 this localization data,  $HP1^{Hsy}$  appears to have a more similar  
 445 pattern to that of Drosophila HP1.

446 To investigate the functional properties of the  $HP1^{Hsa}$   
 447 protein and determine whether it is a functional homologue  
 448 of Drosophila HP1, we generated transgenic *Drosophila* that  
 449 expressed an  $HP1^{Hsa}$ -EGFP fusion gene under the control  
 450 of an *hsp70* heat shock promoter. Homozygous  $HP1^{Hsa}$ -  
 451 EGFP larvae were heat shocked 1 h at 37 °C and allowed to  
 452 recover for 2 h at room temperature. Salivary glands were  
 453 dissected from the larvae, squashed and stained with anti-  
 454 bodies that recognize EGFP and Drosophila HP1. The  
 455 results indicated that  $HP1^{Hsa}$ -EGFP localized to the chro-  
 456 mocenter (the site of fusion of all the centromeres), the

heterochromatic fourth chromosome, and euchromatic sites  
 in a pattern that completely overlapped with endogenous  
 Drosophila HP1 (Fig. 4A). It was possible that co-localiza-  
 tion was due to interactions between the  $HP1^{Hsa}$  CSD and  
 the Drosophila HP1 CSD, since CSDs have been shown to  
 dimerize (Cowieson et al., 2000). Therefore, we assayed the  
 localization of  $HP1^{Hsa}$ -EGFP in larvae lacking endogenous  
 Drosophila HP1.  $HP1^{Hsa}$ -EGFP showed the same pattern of  
 localization on larval polytenes with or without endogenous  
 HP1, indicating that  $HP1^{Hsa}$  associates with chromosomes  
 by similar mechanisms as Drosophila HP1 (Fig. 4B).

Overexpression of Drosophila HP1 enhances silencing of  
 genes repressed by heterochromatin (Weiler and Wakimoto,  
 1995). To test whether  $HP1^{Hsa}$ -EGFP has a similar function,  
 $HP1^{Hsa}$ -EGFP was overexpressed (twofold over endoge-  
 nous HP1) in stocks carrying a tagged *hsp26* heat shock  
 gene inserted at different heterochromatic locations. Expres-  
 sion of  $HP1^{Hsa}$ -EGFP by daily heat shock resulted in a 40%  
 reduction in *hsp26* expression from a centric transgene (Fig.  
 5). Similarly, expression of  $HP1^{Hsa}$ -EGFP resulted in a 50%  
 reduction in *hsp26* expression from a telomeric transgene  
 (Fig. 5). These data demonstrate that the human  $HP1^{Hsa}$   
 protein participates in gene silencing and has similar func-  
 tions as Drosophila HP1.

Several homozygous lethal mutations exist in *Su(var)2-5*,  
 the Drosophila gene encoding HP1 (Eissenberg and Hartnett,  
 1993). These mutations were used to determine whether  
 $HP1^{Hsa}$ -EGFP could rescue lethality. Flies carrying the

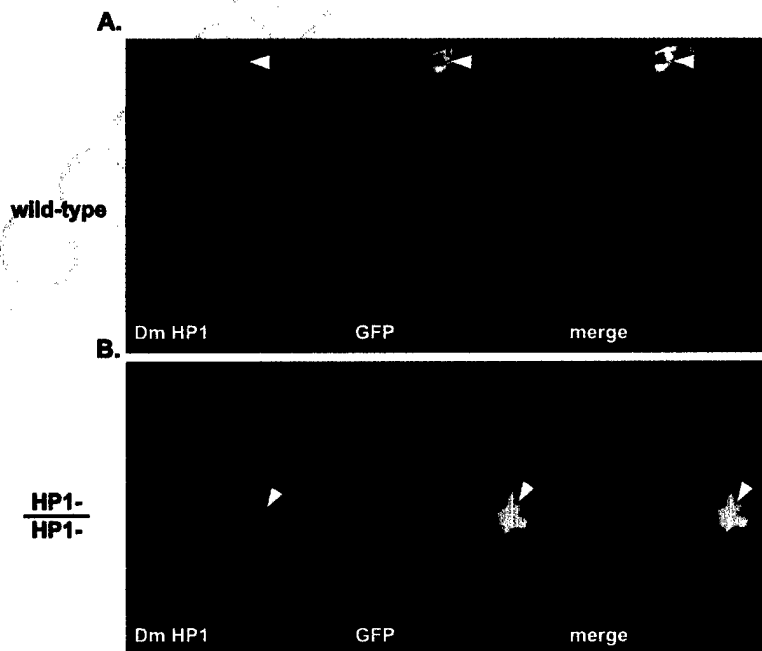


Fig. 4. (A) Polytene chromosomes from third instar larvae salivary glands from a stock carrying an  $HP1^{Hsa}$ -EGFP transgene. The chromosomes were fixed, squashed and stained with antibodies against Drosophila HP1 (DmHP1; red) and GFP (green). The DmHP1 antibody does not recognize  $HP1^{Hsa}$  by Western blot analysis (data not shown). Co-localization is observed in yellow. (B) Polytene chromosomes from HP1 mutants containing the  $HP1^{Hsa}$ -EGFP transgene stained with DmHP1 (red) and GFP (green). Arrowheads denote the chromocenter (formed by the fusion of all centromeres).

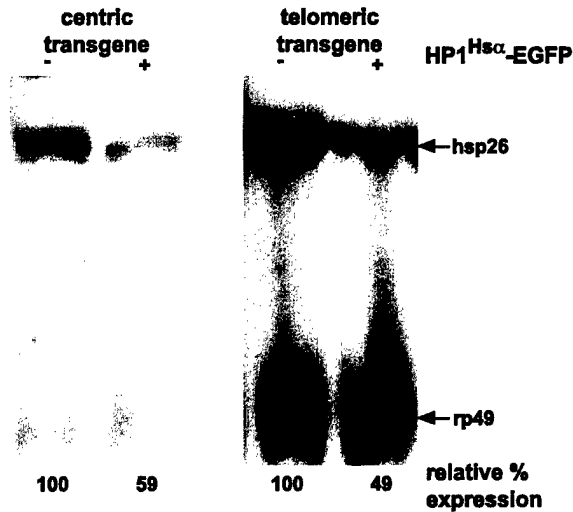


Fig. 5. Effects of  $HP1^{Hsa}$ -EGFP expression on gene silencing. Flies expressing the  $HP1^{Hsa}$ -EGFP transgene were crossed to flies that contain a reporter *hsp26* transgene inserted at a heterochromatic or telomeric location. RNA was isolated from the heatshocked progeny and analyzed by Northern analysis hybridized with radiolabeled sequences corresponding to the *hsp26* reporter gene and the *rp49* loading control.

$HP1^{Hsa}$  transgene that were heterozygous for a mutant allele of the gene encoding HP1 [*Su(var)2-5<sup>04</sup>*] balanced over a chromosome possessing a *Curly* wing mutation were crossed to flies heterozygous for a second mutant allele of the gene encoding HP1 [*Su(var)2-5<sup>02</sup>*] over the *Curly* balancer chromosome. From this cross, *Curly* wing homozygotes, 25% of total progeny, die as early embryos. Flies heterozygous for the *Curly* wing balancer chromosome and a *Su(var)2-5* allele, 50% of the total progeny, were viable. The final class of progeny, heteroallelic for the *Su(var)2-5* mutant alleles, constituting 25% of the total progeny, would be lethal if no rescue is observed. Rescue of such individuals would give rise to straight wing adults. When  $HP1^{Hsa}$ -EGFP was expressed by daily heat shock treatment, 2% (4/218 adults) of the total progeny had straight wings, suggesting partial function of the  $HP1^{Hsa}$ -EGFP fusion protein. More convincing, 14% (35/244 adults) of total progeny were rescued to adulthood by expressing  $HP1^{Hsa}$  without the EGFP tag. These data suggest that despite exhibiting the correct pattern of localization and gene silencing effects, EGFP was limiting the function of  $HP1^{Hsa}$ . The ability of the untagged version to show appreciable rescue (14% vs. 25% for complete rescue) strongly suggests that  $HP1^{Hsa}$  is a functional homolog of the Drosophila HP1 protein.

#### 4. Discussion

##### 4.1. Conservation of gene structure

The *HP1* genomic structure is conserved from Drosophila to humans. Drosophila *Su(var)2-5*, mouse *mHP1 $\alpha$* , *M31*,

and *M32*, and human  $HP1^{Hsa}$ ,  $HP1^{Hsb}$ , and  $HP1^{Hsy}$  are each comprised of five exons and four introns. Translation start begins in exon two in Drosophila *Su(var)2-5*, mouse *mHP1 $\alpha$*  and *M31*, and human  $HP1^{Hsa}$ ,  $HP1^{Hsb}$  and  $HP1^{Hsy}$ . In contrast, the translation start of *M32* is within exon three. Due to the insertion of an intron within exon one, *M32* exon three corresponds to that of exon two in the other HP1 genes (Jones et al., 2001). The CD of all of the mammalian HP1 genes, except for *M32*, is contained in exons two and three (Jones et al., 2001). Exons three and four of *M32* have fused to become exon four. Therefore, the CD of *M32* is found within exons three and four. The CSD of all of the mammalian HP1 genes is found in exons four and five (Jones et al., 2001). The amino acids at the splice junctions are conserved in all the mammalian HP1 family members, except *M32*, but are distinctly different for Drosophila *Su(var)2-5*. Therefore, the genomic structure, but not the intron/exon boundaries, are conserved from Drosophila *Su(var)2-5* to human HP1 family members.

The *Su(var)2-5* gene, at cytological position 28F1-2, encodes a protein that is commonly referred to as Drosophila HP1 and sometimes referred to as HP1a (Smothers and Henikoff, 2001). There are two additional HP1-like genes located at cytological positions 87C and 94C4, called *HP1b* and *HP1c*, respectively. The proteins encoded by these genes do not exhibit a chromosomal distribution pattern that significantly overlaps with HP1 (Smothers and Henikoff, 2001). In addition, these two proteins have limited amino acid sequence identity with HP1; HP1b is 44% identical and HP1c is 31% identical to HP1. Furthermore, *HP1b* and *HP1c* do not have a conserved genomic structure (<http://www.flybase.org/>) with the mammalian members of the HP1 family, as does Drosophila HP1. Thus, HP1 was exclusively used for comparisons in this study.

The three human genes encoding  $HP1^{Hsa}$ ,  $HP1^{Hsb}$  and  $HP1^{Hsy}$  located at 12q13, 17q21 and 7q15, respectively, have several corresponding pseudogenes present in the human genome. In our screen of the human genomic library, we recovered one processed  $HP1^{Hsa}$  pseudogene (data not shown) corresponding to sequences on chromosome 3p11.1 (<http://www.ncbi.nlm.nih.gov/>). Five pseudogenes have been previously published for  $HP1^{Hsb}$ , and four for  $HP1^{Hsy}$  (Jones et al., 2001). The pseudogenes of  $HP1^{Hsb}$ , containing few, if any, introns are found on chromosomes 1q32, 3q26, 14q24, Xp22, and Xq11 (<http://www.ncbi.nlm.nih.gov/>) suggesting they are processed pseudogenes. Eleven  $HP1^{Hsy}$  pseudogenes are found on NCBI Genbank, four of which correspond to the previously published pseudogenes (<http://www.ncbi.nlm.nih.gov/>; Jones et al., 2001). The pseudogenes of  $HP1^{Hsy}$ , containing few, if any, introns are found on chromosomes 2q24, 3p22, 5q22, 6q22.2, 11p11, 11p14, 11q14, 12p13, 12q23, 16p13, and 18p11 (<http://www.ncbi.nlm.nih.gov/>). Thus, each of the functional human HP1 family members is encoded by separate unlinked genes that have multiple pseudogenes scattered throughout the genome.

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571 4.2. *HP1<sup>Hsa</sup>* regulation in breast cancer metastasis

572 *HP1<sup>Hsa</sup>* is down-regulated in highly invasive/metastatic  
 573 breast cancer cells compared to poorly invasive/non-metastatic  
 574 cells (Kirschmann et al., 2000). This regulation likely  
 575 occurs, at least in part, at the transcriptional level and does  
 576 not involve differential DNA methylation. An analysis of  
 577 the DNA sequences in the *HP1<sup>Hsa</sup>* promoter region identified  
 578 potential binding sites for transcriptional regulators that  
 579 might be involved in differential regulation. Only three of  
 580 the binding sites shown in Fig. 3, the two SP1 sites  
 581 immediately upstream of *hnRNPA1*, the CAAT box (position  
 582 –244) and the proximal USF/c-myc site (position  
 583 –109), are conserved between mouse and humans; none  
 584 of the elements can be identified upstream of the gene  
 585 encoding Drosophila HP1.

586 Mutation of a  $\delta$ EF1 binding site (at position –125),  
 587 which associates with the AREB6 protein found to be up-regulated  
 588 in highly invasive/metastatic breast cancer cells (Kirschmann et al., 1999),  
 589 does not appear to be involved in the differential expression of *HP1<sup>Hsa</sup>*.  
 590 Mutation of a c-myc binding site at position +97 and a USF/c-myc site  
 591 at position –109 does not appear to play a role in differential  
 592 regulation either. In contrast, mutation of a USF/c-myc site  
 593 at position –172 abolishes differential regulation. This  
 594 USF/c-myc was protected from DNase I digestion, suggesting  
 595 occupancy by protein(s) in HeLa cells (Biamonti et al., 1993).  
 596 USF/c-myc sites, commonly called E-boxes, are frequently  
 597 bound by a variety of proteins, including USF and Myc family  
 598 members. USF proteins are involved in both gene silencing and  
 599 activation, sometimes at the same site under different  
 600 circumstances (Goueli and Janknecht, 2003). Myc proteins are  
 601 also involved in both gene activation and repression, depending  
 602 on their dimerization partner (Queva et al., 1998). Therefore,  
 603 the function of the distal USF/c-myc site in the differential  
 604 regulation of *HP1<sup>Hsa</sup>* is difficult to predict and warrants  
 605 further investigation.

606 4.3. Function of *HP1<sup>Hsa</sup>*

609 Our data strongly suggest that *HP1<sup>Hsa</sup>* is a functional  
 610 homolog of the Drosophila HP1 protein. The results showing  
 611 that *HP1<sup>Hsa</sup>* can localize to the same sites on polytene  
 612 chromosomes as Drosophila HP1 are consistent with previously  
 613 published results (Ma et al., 2001). We extend these findings  
 614 by demonstrating that *HP1<sup>Hsa</sup>* exhibits the Drosophila HP1  
 615 pattern of localization even in the absence of Drosophila  
 616 HP1. These results suggest a conserved mechanism for  
 617 localization. Previously published results show that  
 618 *HP1<sup>Hsa</sup>* can enhance silencing induced by a transgene  
 619 array in Drosophila (Ma et al., 2001). These arrays have  
 620 similar, yet distinctly different, properties than heterochromatin  
 621 (Prasad-Sinha et al., 2000). Our results clearly demonstrate  
 622 that *HP1<sup>Hsa</sup>* can participate in heterochromatin formation  
 623 and silence euchromatic genes placed within heterochromatin.  
 624 Thus,

*HP1<sup>Hsa</sup>* has gene silencing functions similar to Drosophila  
 HP1.

Species specificity of protein function can be addressed by  
 determining whether a protein from one organism can provide  
 complete function of the homologous protein in another organism.  
*HP1<sup>Hsa</sup>* can rescue the lethality of a Drosophila HP1  
 homozygous mutant; therefore, *HP1<sup>Hsa</sup>* is a functional  
 homolog of Drosophila HP1. In contrast to our findings,  
 the mouse M31 protein was unable to rescue mutant  
 phenotypes associated with *S. pombe* Swi6 mutants (Wang  
 et al., 2000). Rescue was obtained, however, when the  
 Swi6 CSD was substituted for the M31 CSD (Wang et al.,  
 2000). The overall amino acid sequence identity between  
*S. pombe* Swi6 and mouse M31 is 37%. This is much less  
 than the 44% overall amino acid sequence identity between  
 Drosophila HP1 and human *HP1<sup>Hsa</sup>*. In particular, the  
 amino acid sequence identity between the *S. pombe* Swi6  
 CSD and the mouse M31 CSD is 39%, whereas the amino  
 acid sequence identity between Drosophila HP1 CSD and  
 the human *HP1<sup>Hsa</sup>* CSD is 43%. Therefore, the CSD of  
 Drosophila HP1 and human *HP1<sup>Hsa</sup>* is more conserved  
 than the CSD of *S. pombe* Swi6 and mouse M31. The amino  
 acid sequence differences between mouse and *S. pombe* might  
 explain the species-specificity observed.

644 4.4. Model for *HP1<sup>Hsa</sup>* function in breast cancer metastasis

Given the conserved function of *HP1<sup>Hsa</sup>* in gene regulation,  
 one possible role for *HP1<sup>Hsa</sup>* in breast cancer metastasis  
 is gene silencing. Accordingly, the *HP1<sup>Hsa</sup>* gene would be  
 expressed in normal and primary breast cancer tumor cells  
 where it produces protein that functions to silence genes  
 required for invasion and metastasis. In highly invasive/  
 metastatic breast cancer cells, *HP1<sup>Hsa</sup>* expression is  
 reduced and less *HP1<sup>Hsa</sup>* protein is available to carry out  
 gene silencing functions. Clearly in Drosophila and mice  
 HP1 affects gene expression in a dosage-dependent manner  
 (Weiler and Wakimoto, 1995; Festenstein et al., 1999).  
 According to this model, loss of silencing would occur at  
 genes encoding proteins that are required for invasion and  
 metastasis. Therefore, the identification of *HP1<sup>Hsa</sup>* regulated  
 genes is a goal for future investigation.

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## Heterochromatin Protein 1: Development of a Novel Breast Cancer Metastasis Marker

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There was estimated to be 211,300 American women diagnosed with invasive breast cancer in 2003. The five-year survival rate for women with localized breast cancer is 97%, while the five-year survival rate for women with metastatic breast cancer is 23%. Currently, there is a need for molecular markers for metastasis. We have determined that the chromosomal protein, HP1<sup>Hsa</sup>, is down-regulated in metastatic breast tissue compared to primary tumors. Similarly, HP1<sup>Hsa</sup> is down-regulated in highly invasive/metastatic breast cancer cell lines compared to poorly invasive/non-metastatic breast cancer cell lines. We have determined that introduction of exogenous *HP1<sup>Hsa</sup>* by adenoviral infection into a highly invasive/metastatic cell line reduces both the growth rate and the invasive potential. To determine the mechanism of *HP1<sup>Hsa</sup>* down-regulation in highly invasive/metastatic cells, we undertook an analysis of the *HP1<sup>Hsa</sup>* promoter region. Sequence analysis revealed no changes within the promoter region, splice junctions, or coding region of *HP1<sup>Hsa</sup>* in the highly invasive MDA-MB-231 cells compared with the poorly invasive MCF-7 cells. In addition, sequence analysis revealed two CpG islands with the *HP1<sup>Hsa</sup>* promoter region. Bisulfate sequencing detected little DNA methylation throughout these regions in both cell types. Since there were no differences in sequence or methylation status, we hypothesized that differential expression might arise through different interactions between transcription factors and *cis*-acting DNA elements of the *HP1<sup>Hsa</sup>* promoter in the two cell types. We have determined that a USF/c-myc site within the *HP1<sup>Hsa</sup>* promoter is responsible for the differential expression of *HP1<sup>Hsa</sup>* observed between MDA-MB-231 and MCF-7 cell lines. Understanding the regulation and function of HP1<sup>Hsa</sup> in breast cancer metastasis will aid in our general understanding of metastatic progression and may lead to new ways to diagnose and treat metastasis.

# Structural and functional analysis of Heterochromatin Protein 1 in breast cancer cells

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Heterochromatin protein 1 (HP1) is a conserved chromosomal protein enriched in heterochromatic regions of the genome. In humans there are three HP1 family members, HP1<sup>Hsα</sup>, HP1<sup>Hsβ</sup> and HP1<sup>Hsγ</sup>. HP1 proteins contain two conserved domains, an amino chromo domain that associates with methylated lysine 9 of histone H3, and a carboxy chromo shadow domain that interacts with a variety of nuclear proteins. These domains are thought to regulate protein-protein interactions that influence gene expression. HP1<sup>Hsα</sup> is down-regulated in invasive/metastatic breast cancer cells compared with poorly invasive/non-metastatic breast cancer cells. Our working hypothesis is that HP1 plays a role in regulating invasion/metastasis through the regulation of gene expression. To address the *in vivo* function of HP1<sup>Hsα</sup> in human breast cancer, RNAi knock-down experiments are underway. These experiments will determine the effects of reduced HP1<sup>Hsα</sup> on invasion and gene expression. We have also taken a structural approach to understand the mechanism of HP1-mediated gene regulation. Full-length HP1 family members and mutants have been expressed in *E. coli* and purified for X-ray crystallography and biochemical analysis. These studies will allow mechanisms of silent chromatin formation and spreading to be better modeled. This information will be used to understand the role HP1<sup>Hsα</sup> in breast cancer invasion/metastasis.